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13. ABSTRACT (Maximum 200 Words)

Mammary epithelial cells undergo periodic cycles of growth, differentiation and apoptosis during pregnancy and lactation. These processes are initiated by a complex series of signals that include mammotrophic hormones and locally-derived growth factors. This study is aimed at determining the mechanism by which an important mitogenic signal transduction pathway, which is frequently activated in breast carcinoma, inhibits mammary differentiation and apoptosis.

We have demonstrated that the Ras pathway is activated by EGF stimulation of HC11 mammary epithelial cells. This occurs in part via the increase in GTP-bound Ras in the cells. EGF stimulation results in activation of Erk and Akt signal transduction pathways and prevents lactogenic differentiation. Inhibition of either Ras (via DNRas expression) or Erk (via PD98059) or Akt (via wortmannin) can counter the effects of EGF on differentiation. The mechanism of disruption of differentiation appears to involve interference with the growth arrest that occurs prior to the induction of differentiation; the mechanism for growth arrest may require the downregulation of Mek1 expression. The expression of dominant negative Ras in HC11 cells blocked signal transduction to the Ras-Raf-Mek-Erk signal transduction pathway but not signal transduction via the PI-3-kinase pathway.

Expression studies of HC11 cells undergoing lactogenic differentiation using DNA microarrays demonstrated that there is a shift in gene expression that affects genes related to milk production as well as development and growth control.

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INTRODUCTION

Epidemiological studies indicate that the age at first pregnancy and lactation have an impact on later development of breast cancer. Mammary epithelial cells undergo periodic cycles of growth, differentiation and apoptosis during pregnancy and lactation. These processes are initiated by a complex series of signals which include mammotrophic hormones and locally-derived growth factors [1]. This study is aimed at determining the mechanism by which an important mitogenic signal transduction pathway, which is frequently activated in breast carcinoma [2,3], inhibits mammary differentiation and apoptosis.

There are limited human models of mammary differentiation available for study at the present time. However, the HC11 mouse mammary epithelial cells differentiate and synthesize β-casein following growth to confluency and stimulation with the lactogenic hormone mix, DIP (dexamethasone, insulin, prolactin) [4,5]. Regulation of β-casein expression in HC11 reflects *in vivo* regulation of this protein in the mammary gland [4]. Prolactin stimulation results in Jak2-mediated tyrosine phosphorylation of Stat5 a and b and nuclear translocation of the factors [6]. In HC11 cells the activation of Stat5 is not dependent on the Ras-Erk pathway [6] and, in fact, the induction of β-casein expression can be blocked by receptor tyrosine kinase signaling at the time of prolactin addition [7-10]. It is not clear which signal transduction pathways are responsible for the inhibiton of β-casein synthesis by receptor tyrosine kinase signaling. However, the inhibition of β-casein expression by treatment of HC11 cells with EGF or Cripto [CR-1], an EGF family member, occurs through a Ras- and phosphotidylinositol-3-kinase (PI-3 kinase)-dependent mechanism [11]. Determination of the signaling mechanism(s) that are responsible for inhibiting differentiation will provide critical insight into control of this process in HC11 cells. Because inhibition of differentiation in HC11 cells appears to be dependent upon Ras, and possibly its association with PI-3-kinase, these studies focus attention on the role of Ras and its effectors in the differentiation of mammary epithelial tissue. We propose that the growth factor regulated inhibition of DIP-induced differentiation of HC11 cells results from the activation of Ras effector pathways in addition to Raf-Mek-Erk. Inhibition may require activation of the Ras-PI-3-kinase pathway and/or the Ras-RasGAP-Rho pathway.

We will test our hypothesis by constructing HC11 cell lines carrying: effector mutants of Ras which activate only a subset of effector pathways, dominant-negative (DN) mutants of proteins in the Ras-PI-3-kinase and Ras-Rho pathways, and HC11 cell lines expressing elevated levels of enhancers Ras-Raf-Mek-Erk signaling pathway. These cell lines will be used to dissect the control of differentiation using a series of markers for differentiation and cell cycle changes.

cDNA microarray analysis techniques will be used to detect global changes in gene expression induced by differentiation in the HC11 cell background. We will identify genes whose expression is specifically increased and decreased in these cells following induction of lactogenic differentiation. Temporal regulation of the expression of specific genes will be followed during a 72 hour course of lactogenic differentiation.

A complete understanding of the regulation of the differentiation process in mammary epithelial cells will aid in understanding the cellular changes and mechanisms leading to carcinogenesis in this tissue and allow evaluation of therapeutic strategies on the differentiation process.

BODY

The majority of the work completed during this period addressed the goals in the original statement of work as opposed to the revised statement of work for this project. Hence, the results reported here primarily address the original statement of work.

Task 1. Construction of vectors and cell lines. This is described in detail in the manuscript enclosed as Appendix item #1.

Construction of HC11 Tet-Off cell lines. The HC11 cell line was transfected with the pTetOff plasmid (Clontech) and the transfected cells were selected for 10 days with G418 (200-500μg/ml). Then individual colonies were picked, expanded and screened for ability to regulate a Tet-promoter. This was accomplished by transfection with a Tet-promoter luciferase construct and assay for luciferase activity with and without Doxicyclin (0-0.5-2.0μg/ml). Several of the transfected cell lines, Ax-TetOff and C6-TetOff, contained a TRE that could be regulated by Dox. These cell lines (HC11-Tet Off) were used to construct lines for the regulated expression of activated Ras or dominant negative Ras.

Production of Retroviral vector Stocks and infection of HC11 cells. pREV-TRE, a retroviral vector that expresses a gene of interest from Tet-responsive element (TRE), was derived from pLNCX, a Moloney murine leukemia virus (MoMuLV)-derived retroviral vector. The 5' viral LTR controls expression of the transcript that contains φ^+ (the extended viral packaging signal), and the hygromycin resistance (Hyg^r) gene for antibiotic selection in mammalian cells. pRevTRE also includes the *E. Coli* Amp^r gene for antibiotic selection in bacteria. The internal TRE contains seven direct repeats of the 42-bp tetO operator sequence, upstream of a minimal CMV promoter. This promotor was used to inducibly express the genes of interest in response to varying concentrations of Doxicyclin (Dox). TtA binds to the Tet-response element (TRE) and activates transcription from the minimal promoter in the absence of Dox. The plasmids pREV-TRE-RasV12 (active K-Ras 2BV12) and pREV-TRE -RasN17 (Dominant Negative K-Ras 2B(N17) were constructed by introduction of K-Ras cDNA into pREV-TRE.
Retroviral vector stocks of pRev-Tre, pRevTre-RasV12, pRevTre-RasN17 were prepared and used for retroviral infection of HC11-TetOff cells. The HC11-TetOff cell line was infected and selected in hygromycin and Doxicyclin (2μg/ml) for ten days. Six colonies were picked from Tet-Off pREV-TRE, pREV-TRE-RasV12 and pREV-TRE-RasN17 plates and seeded in 24 well plates. These cells was expanded and tested with or without Dox for the presence or absence of Ras RNA by Northern Blot.

Task 2 and 3. Determination of the effect of dominant negative Ras expression on differentiation and Stat5 activation.

EGF blocks hormone-induced HC11 differentiation through Mek and PI-3-kinase-dependent pathways. Previous studies have demonstrated that EGF blocked lactogenic hormone-induced differentiation of HC11 cells [Hynes, 1990 #511], and recent data suggests that this block required Ras and PI-3-kinase activity [DeSantis, 1997 #507]. In the present study specific chemical inhibitors of signal transduction pathways were used to further analyze the contribution of individual signaling pathways to the block of HC11 differentiation by EGF. Because

lactogenic hormone-induced differentiation of HC11 cells is characterized by the initiation of β-casein transcription, the HC11-luci cell line, which contains a β-casein promotor linked to the luciferase gene, was used to provide a rapid readout of the differentiation process.

The HC11-luci cells were induced to differentiate with DIP in the absence and presence of EGF. Specific inhibitors of Mek, and PI-3-kinase were added to cells at the time of induction of differentiation. As expected there was a significant inhibition of β-casein driven luciferase activity in the EGF-treated samples compared to the DIP control. However, several compounds (PD98059, LY294002 and wortmannin) restored the β-casein promotor driven luciferase activity that was blocked by EGF (Figure 1A). The results demonstrated that the inhibition of Mek-Erk signaling by PD98059 and PI-3-kinase signaling by LY294002 and wortmannin disrupted the EGF signaling that inhibited lactogenic hormone-induced differentiation, as measured by the activation of β-casein promotor driven luciferase expression.

The effect of chemical inhibitors of signal transduction pathways on the synthesis of β-casein RNA was examine (Figure 1B). The results confirmed that exposure of HC11 cells to DIP activated β-casein expression and that EGF blocked the expression. However, inclusion of PI-3-kinase or Mek1 inhibitors in the induction media with EGF reversed the EGF-induced inhibition of endogenous on the b-casein promotor activity in the HC11-luci cells.

In addition, the treatment of HC11 cells with DIP resulted in increased Stat5 DNA binding, and previous studies demonstrated that the DNA binding activity of Stat5 was reduced by the simultaneous addition of EGF and lactogenic hormones to HC11 cells [Marte, 1995 #510]. EMSA was performed to examine the ability of the signal transduction inhibitors to alter Stat5 DNA binding. Nuclear extracts were prepared from HC11 cells induced to differentiate in the presence of Jak2, Mek1 or PI-3-kinase inhibitors. The results indicated that prolactin stimulation in the presence of the Mek1 and PI-3-kinase inhibitors enhanced Stat5 binding to DNA compared to the binding detected with prolactin alone (Figure 2A). In contrast, exposure of the HC11 cells to prolactin plus AG490, an inhibitor of Jak2 tyrosine phosphorylation, inhibited Stat5 DNA binding (Figure 2A, lanes 4 and 8). The results in figure 1 indicated that Mek1 and PI-3-kinase inhibitors restored the prolactin-induced Stat5 promotor activity inhibited by EGF. Moreover, the same Mek and PI-3-kinase inhibitors enhanced Stat5 DNA binding. Blocking the Mek-Erk and PI-3-kinase pathways with specific inhibitors both enhanced HC11 differentiation and prevented the EGF-dependent disruption of HC11 differentiation.

HC11 cells expressing dominant negative (N17) Ras exhibit an enhanced differentiation response. Ras activation likely regulates the activation of the Erk pathway by EGF and possibly contributes to the activation of PI-3-kinase. Hence, the role of Ras activation in the disruption of HC11 differentiation by EGF was examined further. HC11 cell clones expressing either activated Ki-Ras (V12) or dominant negative (DN) Ki-Ras (N17) were constructed as described in Materials and Methods. The HC11 cell lines constructed contained the Ras cDNAs under the control of a Tet-responsive promotor in a Tet-off system. Hence, the expression of Ras increased following the removal of doxycycline from the culture media. Several independent clones containing each vector were isolated and characterized for the inducibility of Ras gene expression following the removal of doxycycline from the cultures. As expected, the inducibility varied for the individual Ras(V12) and DN Ras clones. The results obtained with three independent clones derived from each vector are shown in Figure 3.

The DN Ras and the Ki-Ras(V12) HC11 cell lines were compared to the vector control cell line, REV-TRE, to determine the effect of the Ras gene expression on lactogenic hormone-

induced differentiation. HC11 transfectant cell lines expressing dominant negative Ras(N17) or activated Ras(V12) along with the vector control cell line were grown for 72 hours in the absence of doxycycline at which point the confluent cultures were incubated in DIP differentiation media. RNA was harvested from cells at 0, 48, and 72 hours post addition of DIP and used to determine the level of Ras and β casein expression by Northern blotting. The results in Figure 3 indicated that Ki-Ras(V12) expression inhibited β-casein expression by approximately 50% compared to the TRE control cell line. In contrast, the expression of dominant negative Ras(N17) enhanced β-casein induction up to two-fold compared to the control. The results demonstrated that the amount of N17 Ras expression correlated with the effect on differentiation. The HC11 cell clone expressing the greatest amount of Ras N17 (clone 12) exhibited the greatest level of β-casein expression.

In parallel experiments the effect of Ras expression on the prolactin-induced tyrosine phosphorylation of Jak2 and Stat5 was examined. HC11 TRE vector control cells as well as the Ki-Ras(V12) clone 1 and DN Ras(N17) clone 12 cells were stimulated with prolactin and the phosphorylation status of the Stat5 protein was determined by immunoprecipitation and Western blotting using anti Stat5 tyrosine 694 (Y694) phosphorylation site-specific antibodies. The results, seen in Figure 4A, indicated that the tyrosine phosphorylation of Stat5 was enhanced and sustained in the DN Ras(N17) HC11 cell line compared to the TRE vector control cell line. However, the tyrosine 694 phosphorylation was of a shorter duration in the cell lines expressing activated Ki-Ras(V12) than in the TRE control cells. These results suggested that Ras-dependent signal transduction can modulate Stat5 phosphorylation in HC11 cells in response to prolactin. The Stat5 EMSA results supported this conclusion (Figure 4B). Enhanced Stat5 DNA binding in response to prolactin stimulation was observed in the DN Ras(N17) HC11 cell lysates as compared to the vector control. In contrast, the Stat5 DNA binding activity was reduced in cells expressing activated Ki-Ras(V12). In conclusion, an increase in HC11 cell lactogenic hormone-induced differentiation is observed following blockade of the Ras signaling pathway. Moreover, in the HC11 cells that have Ras activity blocked, the enhancement of hormone-induced differentiation appeared to be attributable to an increase in Stat5 tyrosine phosphorylation and to an increase in Stat5 DNA binding resulting in enhanced transcription of β-casein, a Stat5-regulated gene.

Infection of HC11 cells with DN Ras adenovirus enhances lactogenic differentiation. Infection of cells with replication defective adenovirus encoding dominant negative Ha-Ras(N17) was used as another mechanism to examine the influence of the Ras pathway on lactogenic differentiation. HC11 cells and HC11-luci cells were infected with 10 MOI of either replication defective control adenovirus or adenovirus encoding DN (N17) Ras. At 24 or 48 hours post infection the cells were examined for the effect of DN Ras on Stat5 phosphorylation, β casein promotor activity and β casein RNA levels. As demonstrated in Figure 5A HC11-luci cells infected with control virus or DN Ras virus were stimulated with DIP and the level of Stat5 tyrosine 694 phosphorylation was determined. The results indicated that the expression of DN Ras (N17) increased the level of Stat 5 phosphorylation in response to DIP compared to either uninfected or vector control-infected cells. HC11-luci cells infected with either replication defective control adenovirus or adenovirus encoding DN Ras (N17) were tested for activation of β-casein promotor-driven luciferase activity (Figure 5B). There was a five-fold increase in the activation of luciferase activity in the DN Ras (N17) cells compared to the uninfected cells or the control adenovirus infected cells. In addition, there was some activation of luciferase activity in cells

infected with the DN Ras (N17) virus without DIP exposure. This result was reproducible and is not seen when uninfected cells or vector infected cells were exposed to DIP. Finally, RNA from HC11 cells infected with either replication defective control adenovirus or adenovirus encoding DN Ras (N17) was tested for expression of β-casein following exposure to DIP for 24 or 48 hours. The results in Figure 5C indicated that the infection with DN Ras (N17) virus resulted in a two-fold increase in β-casein RNA compared to the uninfected or vector infected cells exposed to DIP.

HC11 cells expressing dominant negative (N17) Ras exhibit reduced response to EGF. Studies were performed to determine if the DN Ras (N17) expression could block EGF-induced responses in stable transfectants of HC11 cells. HC11 cells respond mitogenically to EGF. The TRE vector control cells and the DN Ras (N17) cells were stimulated with EGF and the ability of the cells to proliferate was examined using the MTT assay. The results demonstrated that the DN Ras (N17) cell line was growth inhibited by 40% in both the absence and presence of EGF compared to the vector control cell line. This experiment was repeated using TGFα treatment of HC11 vector control and DN Ras (N17) cells. Again, the DN Ras (N17) cells exhibited a lower response to EGF and TGFα than did the vector control cell line. (Figure 6)

The ability of DN Ras to prevent the disruption of lactogenic hormone-induced differentiation by EGF in HC11 cells was examined. The cells were exposed to lactogenic hormone differentiation media in the presence and absence of EGF for varying lengths of time, RNA was extracted and the level of β-casein mRNA was analyzed by Northern blotting. The results in Figure 6 demonstrated that EGF did not inhibit the induction of β-casein transcription in response to DIP treatment in the DN Ras (N17) cell line and, hence, it appeared that differentiation proceeded in these cells even in the presence of EGF. In contrast, the vector control cell line did not express β-casein RNA in the presence of DIP plus EGF. These results demonstrated that DN Ras expression prevented the disruption of hormone-induced differentiation by EGF in HC11 cells.

HC11 cells expressing dominant negative (N17) Ras exhibit reduced Erk activation in response to EGF.

HC11 cells expressing DN Ras(N17) were examined to determine if expression of DN Ras prevented the activation of Mek-Erk or PI-3-kinase signaling in response to EGF. In Figure 7 the stable transfectants were removed from doxycycline and grown to confluence. The cells were starved and then stimulated with EGF for varying amounts of time. Cell lysates were prepared and analyzed by Western blot using antibodies that detect phosphorylated forms of different signaling proteins. The results revealed that stimulation of HC11 vector control cells with EGF resulted in activation of p44Erk as detected by reactivity with an antibody that recognizes the active phosphorylated form of Erk. In contrast, in HC11 cells expressing DN Ras (N17) there was no activation of p44Erk, although the Erk protein levels in the cells were similar to those in the vector control cells. The analysis of other signaling proteins revealed that Akt was activated in the control HC11 cells and partially attenuated in the DN Ras HC11 cells following treatment with EGF. This demonstrated that the PI-3-kinase pathway was not completely blocked by DN Ras expression in HC11 cells. Moreover, activation of Jun kinase and p38 kinase by EGF was not deficient in the N17 Ras HC11 cells (data not shown). These results suggest that the Mek-Erk pathway was most sensitive to inhibition by DN Ras expression.

Cells infected with the control adenovirus vector or adenovirus encoding DN Ras (N17) were examined for the effect of EGF on signal transduction pathways in an analogous fashion. The results in Figure 7 demonstated that DN Ras (N17) adenovirus also blocked the activation of Erk but not the phosphorylation of AKT on serine 473, used as a measure of PI-3-kinase activity. The results from the DN Ras(N17) expressing cells indicates that blocking the Ras pathway in this manner in HC11 cells primarily blocks signaling to the Raf-Mek-Erk pathway. Hence, these data support the conclusion that in HC11 cells activated Ras(V12) inhibits β-casein transcription via Mek-Erk signaling, and that the effect of DN Ras(N17) expression on β-casein is primarily a result of its inhibition of the Mek-Erk pathway.

Task 5. DNA Microarray analysis of changes in gene expression following induction of lactogenic differentiation.

Cell preparation.
HC11 mouse mammary epithelial cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, 5 μg/ml Insulin, 10 mM Hepes and 10 ng/ml epidermal growth factor(EGF). Cells were maintained in T75 flasks after confluence for 4 days, then starve the cells in the media without EGF for 24 hours. The cells were then incubated in differentiation media(serum containing RPMI with dexamethazone(10^{-6} M), insulin(5 μg/ml) and prolactin(5 μg/ml) for 72 hours, undifferentiated HC11 cells were used as control. The cells were scraped from the flasks and precipitated for microarray RNA extraction.

RNA preparation
RNAs were extracted using Trizol reagent (Invitrogen) and RNeasy maxi kit (Qiagen). Wash the cells in the flask once with PBS. Add 5 ml of Trizol to a 75 cm^2 flask (about 2 $x10^7$ cells) and mix by rotating. Add 2/10 volume of chloroform and shake for 15 seconds. Centrifuge at 12,000g for 15 minutes at 4°C. Take off the supernatant and add it to a polypropylene tube, recording the volume of the supernatant. Then 0.53 volumes of ethanol were added to the supernatant slowly while vortex, this step produceed a final ethanol concentration of 35%. Add the supernatant from an extraction to an RNeasy maxi column, which is seated in a 50 ml centrifuge tube. Centrifuge at 2880g in a clinical centrifuge with a horizontal rotor at room temperature for 5 minutes. Pour the flow-through back onto the top of the column and centrifuge again. Discard the flow-through and add 15 ml of RW1 buffer to the column, centrifuge at 2880g for 10 minutes. Discard flow-through then add 10 ml of RPE buffer and centrifuge at 2880 g for 10 minutes. Discard flow-through and add another 10 ml of RPE buffer and centrifuge at 2880g for 15 minutes. Put the column in a fresh 50 ml tube and add 1 ml of DEPC treated water from the kit to the column and lets stand for 1 minute, centrifuge at 2880g for 5 minutes. Repeat this process once. Concentrate samples to greater than 1 mg/ml by centrifugation on a MicroCon 100 filter unit at 500g. Determine the concentration and ratio of RNA in the concentrated sample by spectrophotometry. Store at -80°C. Or purify RNA to get mRNA using Oligtex mRNA kit.

Labeling, hybridization and analysis
Gene expression analysis was performed by Atlas Glass Mouse 3.8 Microarrays(Clontech Laboratories), which include 3800 mouse DNA oligo probes, a list of these genes is available at the Clontech web site (http://www.clontech.com/atlas/genelist/index.shtml). In addition, mouse NIA(15K) slides were used for microarray experiments. Fluorescent labeling of RNAs was

performed by using an Atlas Glass fluorescent labeling kit (Clontech Laboratories) according to manufacturer's manuals. Synthesized first-strand cDNAs from RNA of HC11 cells with and without differentiation were labeled with fluorescent dyes, Cy3 and Cy5 (Amersham Pharmacia Biotech), respectively. The labeling was switched during experiment, i.e. differentiation group was labeled with Cy3 two times, and Cy5 two times; and the control group was labeled with Cy5 two times, and Cy3 two times, vise versa. The quality of the labeling and the amount of each probe used were determined by absorbance measurement for Cy3 and Cy5 probes in a Beckman DU-600 scanner. Hybridization of the microarrays was carried out in a hybridization solution for 16 hours at 50°C. Then wash the slide with wash solution for 3 times provided by manufacturer. The microarray slides were scanned and analyzed by using a GenePix 4000B scanner in both Cy3 and Cy5 channels. The differentiation induced gene up- or down-regulations were obtained by dividing differentiation value over control value. The average of Cy3 and Cy5 signals from nine house-keeping genes gives a ratio which was used to normalize the individual signals. The data is included as Figure 8.

Generation of probes

Using accession number of interested gene to find out the mRNA sequence at internet, design primers for RT-PCR about 200-500 bp gene which can be used as a probe. Use Gene Amp RT-PCR kit (Roche) to amplify the cDNA and insert the correct-sized fragment into a pCR2.1 TA cloning kit(Invitrogen), candidate clone was sent to sequencing to prove the correct sequence. Double strand DNA of the insert was digested from pCR 2.1 plasmid, gel purified as a probe. The probes were used for hybridization to Northern blots containing RNA from HC11 cells undergoing lactogenic differentiation.

Verification of gene expression by Northern blot.

For Northern blot experiment, HC11 cells were treated same as microarray experiment, and then differentiated for 12 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h, respectively, undifferentiated cells at 0 h, and 144 h were used as controls. RNA samples (10 μg) were electrophoresed on agarose gels and transferred to nylon filters. The filters were reacted with labeled probes in hybridization solution and incubated overnight. The blots were washed and expose to X-ray film and then quantitated on a beta scanner. Beta-actin probe was used to hybridize the same membrane and then scanned to get a normalized data. Genes that exhibit increases in expression during differentiation are shown in figure 9 and genes that are decreased in figure 10.

KEY RESEARCH ACCOMPLISHMENTS

-Construction of HC11 mouse mammary epithelial cells capable of regulating a tetracycline-inducible promotor.

-Construction of HC11 cell lines expressing RasV12 and RasN17 under the control of a regulatable promotor.

-Demonstartion that EGF disrupts differentiation via stimulation of the Erk and Akt pathways.

-Demonstration that EGF stimulation results in accumulation of GTP-bound Ras.

-Demonstration that DNRas adenovirus can be used to infect HC11 cells and that DNRas expression enhances activation of the β casein promotor.

-Detection of a set of genes that is expressed at 2-fold or greater levels during lactogenic differentiation of HC11 cells.

- Detection of a set of genes that is expressed at 2-fold or lower level during lactogenic differentiation of HC11 cells.

REPORTABLE OUTCOMES

- Construction of HC11 mouse mammary epithelial cells capable of regulating a tetracycline-inducible promotor.

CONCLUSIONS

We have demonstrated that the Ras pathway is activated by EGF stimulation of HC11 mammary epithelial cells. This occurs in part via the increase in GTP-bound Ras in the cells. EGF stimulation results in activation of Erk, Akt and other signal transduction pathways and prevents lactogenic differentiation. Inhibition of either Ras (via DNRas expression) or Erk (via PD98059) or Akt (via wortmannin) can counter the effects of EGF on differentiation. The mechanism of disruption of differentiation appears to involve interference with the growth arrest that occurs prior to the induction of differentiation; the mechanism for growth arrest may require the downregulation of Mek1 expression. In addition, EGF mitogenic stimulation also inhibits Stat5 binding to its DNA binding site in the β casein promotor.

This data focuses on the role of two Ras effector signal transduction pathways (Erk and Akt) in preventing mammary epithelial cell differentiation. Our results indicate that inhibition of either or both of the pathways blocked the disruption of differentiation by mitogens of the EGF family. However, the block in signal transduction that resulted from dominant negative Ras expression inhibited the Mek-Erk signal transduction pathway and this inhibition is responsible for the effect on lactogenic differentiation. This approach to regulating differentiation may be useful in designing therapeutic approaches using signal transduction inhibitors (STIs).

A list of genes transcriptionally regulated during lactogenic differntiation has been identified. The changes in the level of RNA for these genes has been confirmed by Northern blotting. Using this list and additional data from future expression profiling experiments, novel pathways important to the regulation of lactogenic differentiation will be identified.

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APPENDIX

The figures cited in the body of the report and the figure legends are contained in the attached Appendix.

Figure Legends

Figure 1. **A.** The effect of signal transduction inhibitors on EGF disruption of differentiation. HC11-luci cells were grown to confluence in EGF-containing media then induced to differentiate in DIP-induction media in the presence or the absence of EGF (10ng/ml). Inhibitors were added at the time of DIP induction at previously determined optimal concentrations (PD98059-20μM, LY294002-10μM, wortmannin-100nM). The luciferase activity in lysates was determined at 48 hours post induction. Luciferase activity was normalized to cell protein. The results, presented as luciferase activity in relative units and represent the mean of six determinations. *These values represent statistically significant difference (p-value .001) from the DIP + EGF condition. **B.** The effect of signal transduction inhibitors on EGF disruption of β-casein transcription in HC11 cells. The HC11 cells were induced to differentiate in DIP-induction media with and without EGF (10ng/ml). Inhibitors were added at the time of induction at slightly lower than optimal concentrations to avoid toxicity (PD98059-10μM, LY294002-5μM, wortmannin-50nM).. Total cell RNA was harvested at 48 or 72 hours after transfer to DIP-induction media. β-casein induction was determined via Northern Blot. For quantitation β-casein expression at 48 hours was normalized to β-actin. The results are presented as relative units (R.U.).

Figure 2. EMSA. The effect of inhibitors on Stat5 DNA binding. **A.** HC11 cells were grown to confluence in EGF-containing media, then incubated in EGF-free media for three days and serum-free media for 1 day. HC11 cells were pretreated with specific kinase inhibitors for 2 hours prior to DIP-induced differentiation for 15 minutes in the presence of the inhibitors. Nuclear lysates were prepared and used for Stat5 binding to the β-casein GAS element in the presence or absence of anti-Stat5 antibody. Lanes 1 and 5, control (DIP alone); lanes 2 and 6, PD 98059 (20 μM) plus DIP; lanes 3 and 7, wortmannin (20 nM) plus DIP; lanes 4 and 8, AG 490 (20 μM) plus DIP. Lane 5, 6, 7, 8 the binding was performed in the presence of anti-Stat 5 antibody for supershift. Lane 9, 10 the samples were the same as lanes 1, 2 but rabbit IgG was added. **B.** Gel shift (control) using Sp1 oligos as a loading control. The same protein lysates were used as in Part A, but the binding was to an Sp1 oligonucleotide. SP: supershift of Stat5.

Figure 3. HC11 cells expressing activated Ki-Ras (V12) and DN-Ki Ras (N17) under the control of the Tet-responsive promotor were utilized to evaluate the effect of Ras-based signal transduction on lactogenic differentiation. Three individual clones of HC11 cells expressing either Ras V12 or Ras N17 under the control of the Tet responsive promotor were grown to confluence, incubated in the absence of doxycycline and exposed to DIP differentiation media. The vector control cell line, TRE, was treated in parallel. RNA was harvested from cells at 0, 48, 72 and 96 hours after addition of DIP and used to determine the level of Ras and β-casein expression by Northern blotting. The Ras and β-casein expression was quantitated using a beta scanner and were normalized to the actin signal and reported in relative units.

Figure 4. The effect of RasV12 and RasN17 expression on Stat5 phosphorylation and DNA binding. **A.** HC11 TRE vector control cells and HC11 cell lines expressing activated RasV12(1) or DNRasN17(12) were grown to confluence in EGF-containing media without doxycycline to induce the expression of Ras. The cells were stimulated with DIP, and nuclear extracts were prepared from cells at 0, 15 minutes, 1 hour and 24 hours post stimulation. Total Stat5 was

immuno-precipitated and analyzed by western blotting with antibodies for phospho-Stat5 or total Stat5. The amount of phospho-Stat5 and total Stat5 on the western blots was quantitated using a CCD camera, and the amount of phospho-Stat5 was normalized to the total Stat5 and reported in relative units. **B.** EMSA. HC11 TRE vector control, RasV12(1), and RasN17(12) cells were grown to confluence in EGF-containing media, then incubated in EGF-free media for three days and serum-free media for 1 day. Treated cells were exposed to differentiation media for 15 minutes and control cells (T=0) were not exposed to DIP. **(left panel)** Nuclear lysates were prepared and used for Stat5 binding to the β-casein GAS element in the presence or absence of anti-Stat5 antibody as indicated. Lanes 1, 4, 7, TRE control; lanes 2, 5, 8, RasV12(1); lanes 3, 6, 9, RasN17(12). **(right panel)** Sp1 binding oligonucleotides were used as a loading control. Lanes 1, 4, 7 TRE control; lanes 2, 5, 8 RasV12(1); lanes 3, 6, 9 RasN17(12). Lanes 1,2,3 contain contol lysate; lanes 4, 5, 6 contain lysate from DIP-treated cells; lanes 7, 8, 9 contain lysate from DIP-treated cells with the addition of 50 X cold Sp1 oligonucleotides. SP: Stat5 supershift.

Figure 5. The effect of dominant negative Ras (N17) adenovirus expression on lactogenic differentiation in HC11 cells. **A**. The effect of DNRas (N17) adenovirus on Stat5 phosphorylation in response to lactogenic hormone was determined. Uninfected HC11 cells, HC11 cells infected with a control adenovirus vector and HC11 cells infected with adenovirus encoding DN Ras (N17) (at M.O.I. = 10) were incubated for 24 hours; the cells were then serum-starved overnight and stimulated with DIP for 7.5 minutes. Total Stat5 was immunoprecipitated and analyzed by western blotting with antibodies for phospho-Stat5 or total Stat5. The amount of phospho-Stat5 and total Stat5 was quantitated using a CCD camera, and the amount of phospho-Stat5 was normalized to the total Stat5 and reported in relative units.
B. HC11-luci cells infected with adenovirus vector control or adenovirus encoding dominant negative Ras (N17) were used to determine the effect of N17 Ras on β-casein driven luciferase activity. The cells were infected with the viruses described above and incubated for a period of 24 hours in media without EGF. The cells were then either stimulated with DIP for 24 hours or incubated in media without EGF for an additional 24 hours. The luciferase activity in lysates was determined and normalized to cell protein; the results, presented as luciferase activity in relative units, represent the mean of four determinations. **C**. The effect of dominant negative Ras adenovirus infection on HC11 expression of β-casein was determined. The HC11 cells were infected with the control or dominant negative Ras (N17) virus as described above. RNA was isolated at 0, 24 and 48 hours post induction of differentiation and used to determine the amount of β-casein transcription by Northern blotting. Hybridization of the blots with an actin probe was used as a control for RNA loading. The expression of the β-casein RNA was quantitated by measurement on a β-scanner, normalized to actin and expressed on a relative scale.

Figure 6. Ras N17 expression inhibits EGF-induced proliferation and prevents EGF-dependent disruption of lactogenic differentiation. **A**. HC11 TRE vector control and RasN17 (12) cells were grown in absence of doxycycline and then seeded in microtiter plates in 0.5% serum-containing media with and without EGF (10μg/ml). Cell proliferation was determined at 24, 48, 72, 96 hours post addition of EGF using the MTT assay. The results are reported as the mean of four determinations. **B**. The HC11 TRE vector control and RasN17 (12) cells were grown as

described above and exposed to EGF (10ng/ml) or TGFa (10ng/ml). Cell proliferation was determined using the MTT assay and the results represent the mean of four determinations. **C.** HC11 TRE vector control and RasN17 (12) cells were grown to confluence in absence of doxycycline and then exposed to DIP in the presence or absence of EGF (10ng/ml). Total RNA was isolated after 72 hours and used for Northern blotting. The blots were hybridized to probes for β-casein and actin. The β-Casein and actin RNA was quantitated using a beta scanner; the β-casein RNA was normalized to the actin RNA. The % reduction of β-casein RNA by the addition of EGF during DIP-induced differentiation was calculated using the values for normalized β-casein expression.

Figure 7. The effect of dominant negative Ras (N17) adenovirus expression on signal transduction pathways in HC11 cells. **A**. The HC11 TRE vector control cells and RasN17 cell lines were grown to confluence in EGF-containing media lacking doxycycline. The cells were incubated in media without EGF or media without EGF and serum (*) prior to restimulation with EGF (100ng/ml) for the time indicated. Lysates of cells were harvested and analyzed by Western blotting using antibodies specific for phosphorylated and nonphosphorylated forms of the indicated proteins. **B**. HC11 cells infected with control adenovirus vector or DN Ras (N17)-encoding adenovirus at an MOI of 10 were incubated in serum-containing media for 24 hours and incubated in EGF-free media for 20 hours prior to stimulation with EGF (100ng/ml) for the time indicated. Lysates of cells were harvested and analyzed by Western blotting as in part A.

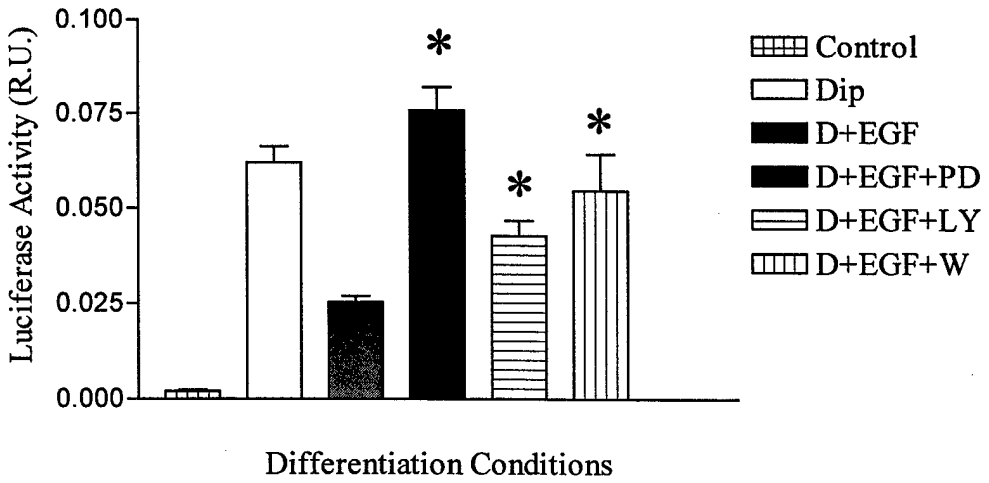
Figure 8. The gene expression pattern in differentiated HC11 cell by using DNA microarray technique. Two different arrays of cDNAs were used for the study. The results shown here include a list of genes expressed at >1.5 fold increased levels or >1.5 fold decreased levels following lactogenic differentiation of HC11 cells.

Figure 9. Northern Blot of HC11 RNA following induction of lactogenic differentiation. RNA was isolated from HC11 cells induced to diffentiate with lactogenic hormone at various time post hormone addition. The blots were hybridized with cDNA probes from genes that were up-regulated as determined by array analysis. Quantitation of the hybridization results was performed and normalized to the actin signal.

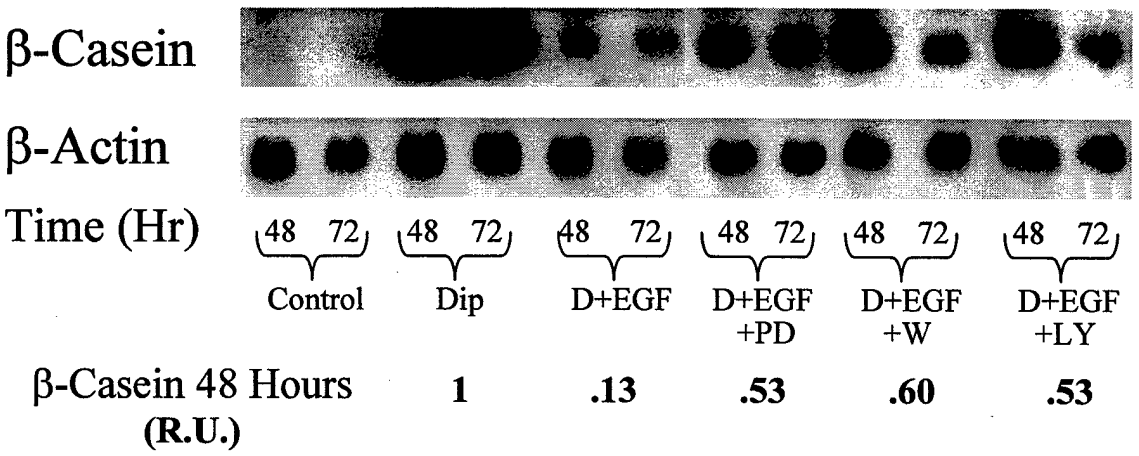
Figure 10. Northern Blot of HC11 RNA following induction of lactogenic differentiation. RNA was isolated from HC11 cells induced to diffentiate with lactogenic hormone at various time post hormone addition. The blots were hybridized with cDNA probes from genes that were down-regulated as determined by array analysis. Quantitation of the hybridization results was performed and normalized to the actin signal.

A.

FIG. 1



B.



β-Casein 48 Hours (R.U.)	1	.13	.53	.60	.53

FIG.2

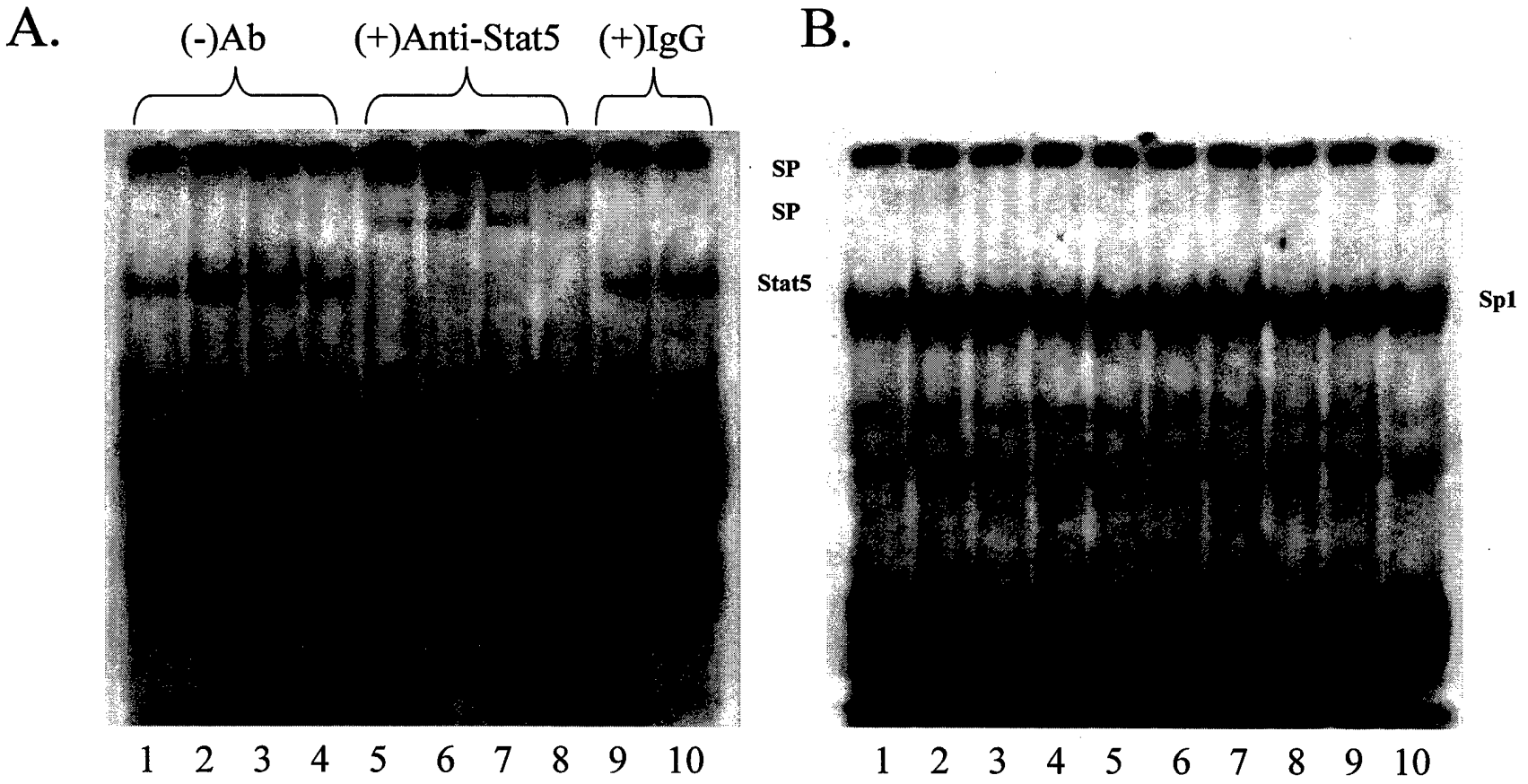

A.
(-)Ab
(+)Anti-Stat5
(+)IgG
SP
SP
Stat5
1 2 3 4 5 6 7 8 9 10
B.
Sp1
1 2 3 4 5 6 7 8 9 10

FIG 3

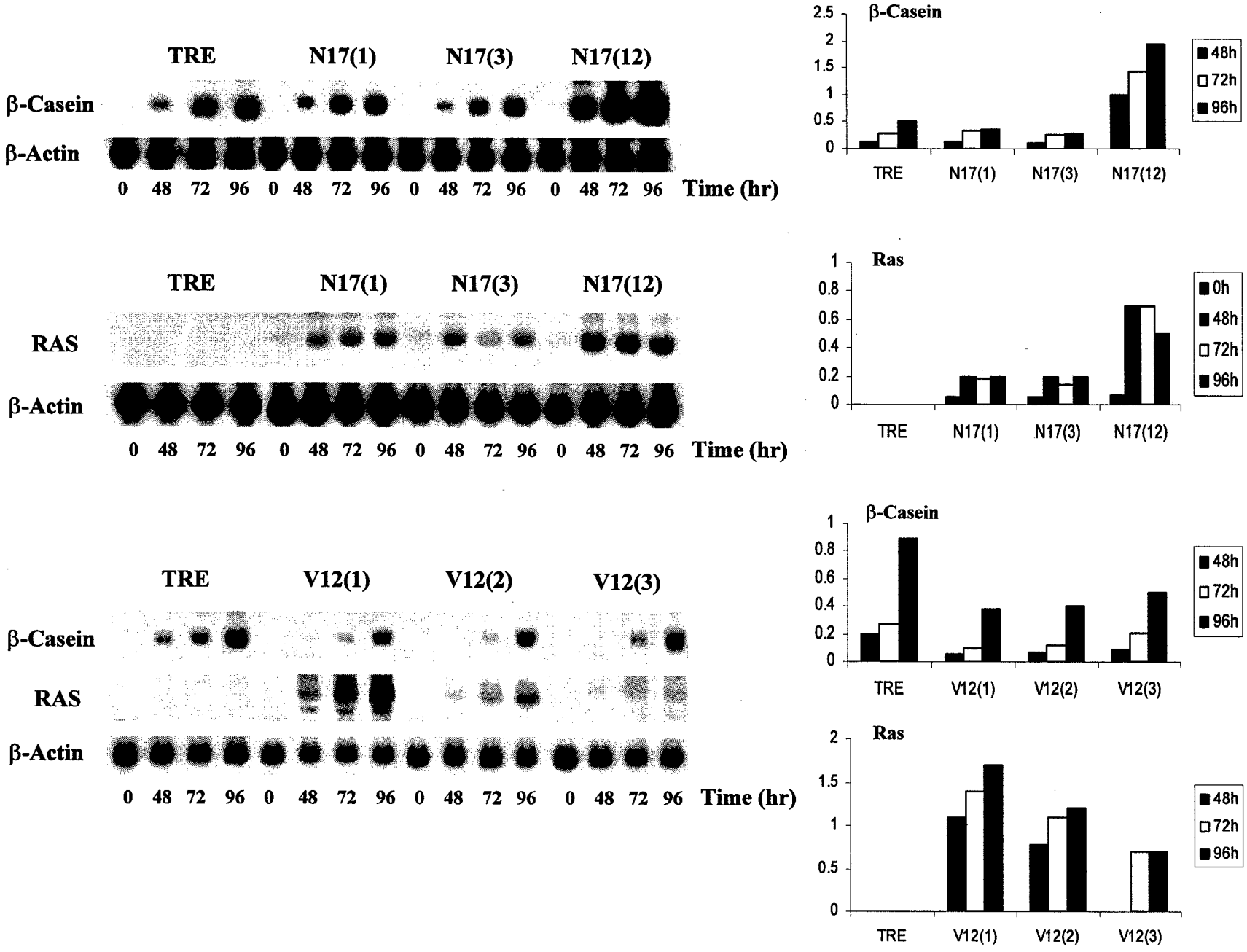

TRE
N17(1)
N17(3)
N17(12)
β-Casein
β-Actin
0 48 72 96 0 48 72 96 0 48 72 96 0 48 72 96 Time (hr)
β-Casein
2.5
2
1.5
1
0.5
0
TRE
N17(1)
N17(3)
N17(12)
48h
72h
96h
TRE
N17(1)
N17(3)
N17(12)
RAS
β-Actin
0 48 72 96 0 48 72 96 0 48 72 96 0 48 72 96 Time (hr)
Ras
1
0.8
0.6
0.4
0.2
0
TRE
N17(1)
N17(3)
N17(12)
0h
48h
72h
96h
TRE
V12(1)
V12(2)
V12(3)
β-Casein
RAS
β-Actin
0 48 72 96 0 48 72 96 0 48 72 96 0 48 72 96 Time (hr)
β-Casein
1
0.8
0.6
0.4
0.2
0
TRE
V12(1)
V12(2)
V12(3)
48h
72h
96h
Ras
2
1.5
1
0.5
0
TRE
V12(1)
V12(2)
V12(3)
48h
72h
96h

FIG.4

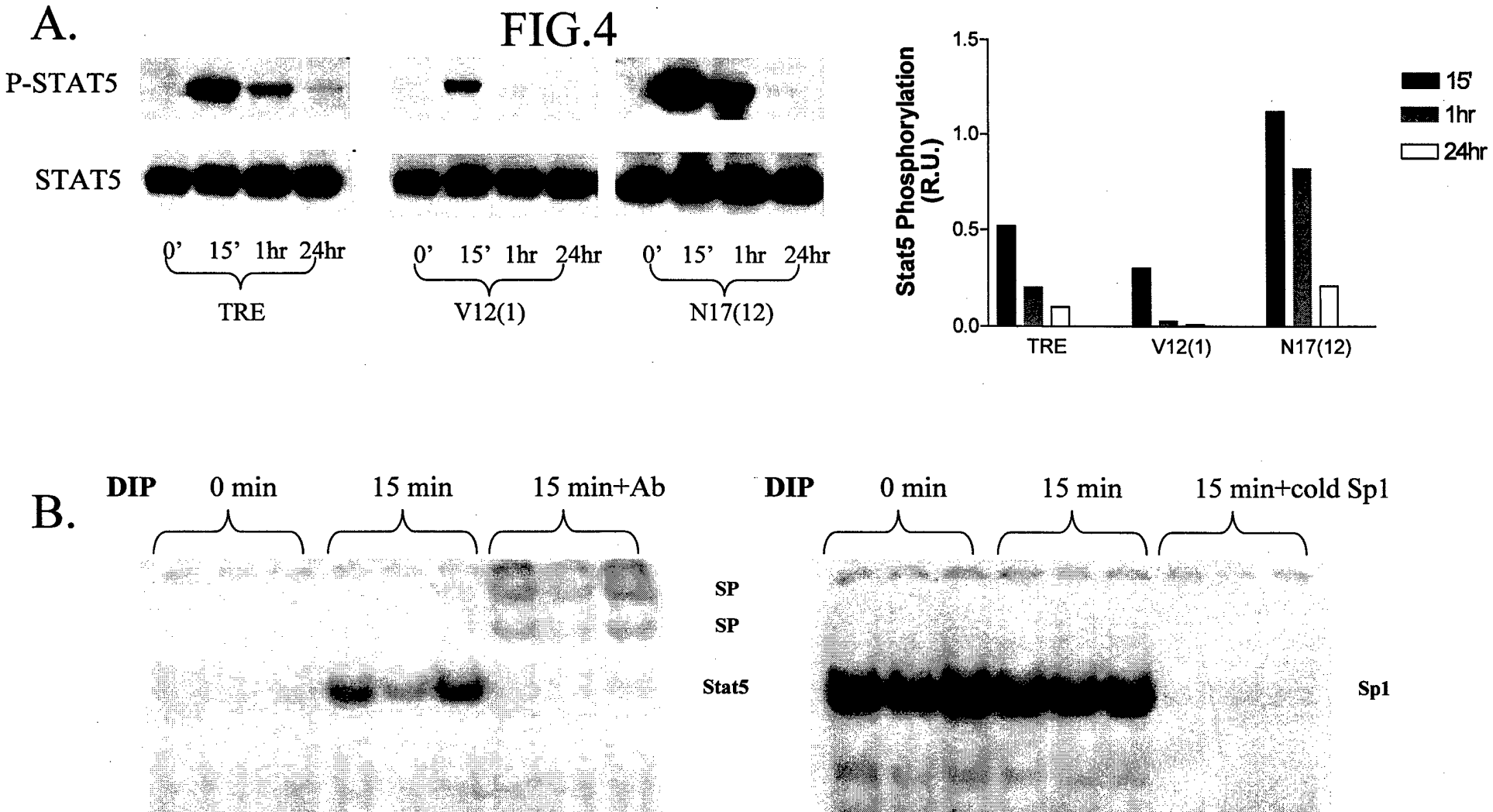
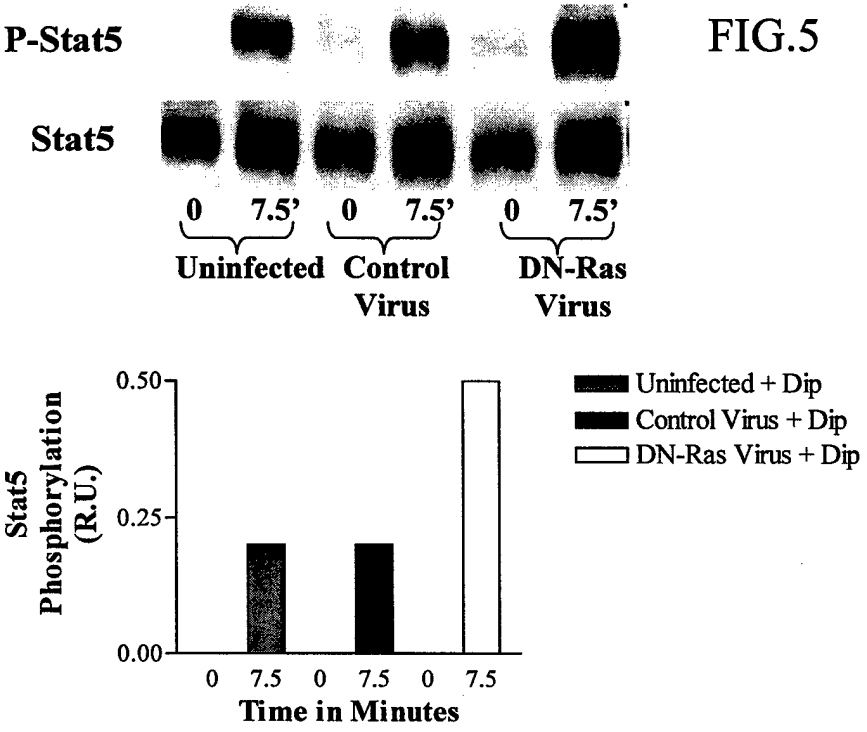
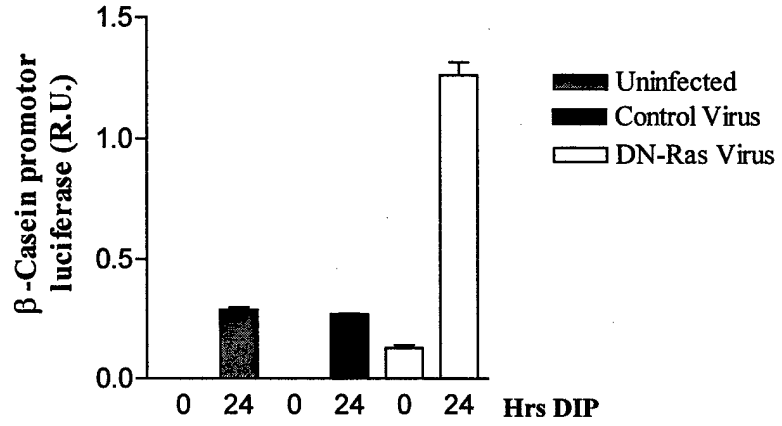

A.
P-STAT5
STAT5
0' 15' 1hr 24hr
TRE
0' 15' 1hr 24hr
V12(1)
0' 15' 1hr 24hr
N17(12)
Stat5 Phosphorylation (R.U.)
1.5
1.0
0.5
0.0
TRE
V12(1)
N17(12)
15'
1hr
24hr
B.
DIP
0 min
15 min
15 min+Ab
SP
SP
Stat5
1 2 3 4 5 6 7 8 9
DIP
0 min
15 min
15 min+cold Sp1
Sp1
1 2 3 4 5 6 7 8 9

FIG.5

A.


P-Stat5
Stat5
0 7.5' 0 7.5' 0 7.5'
Uninfected
Control Virus
DN-Ras Virus
Stat5 Phosphorylation (R.U.)
0.50
0.25
0.00
0 7.5 0 7.5 0 7.5
Time in Minutes
Uninfected + Dip
Control Virus + Dip
DN-Ras Virus + Dip

B.


β-Casein promotor luciferase (R.U.)
1.5
1.0
0.5
0.0
0 24 0 24 0 24
Hrs DIP
Uninfected
Control Virus
DN-Ras Virus

C.

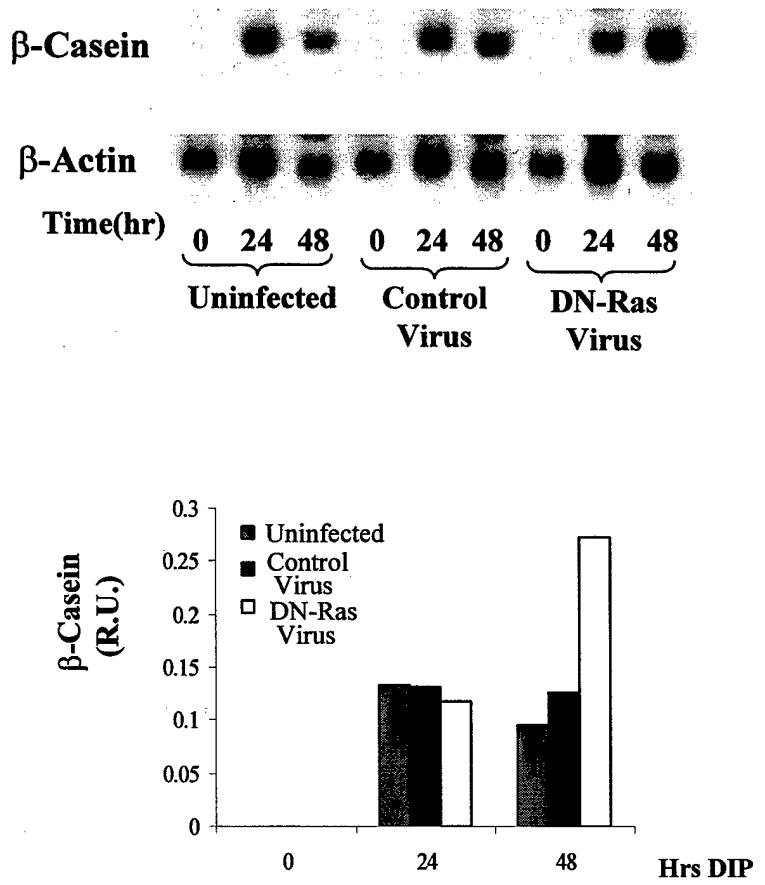
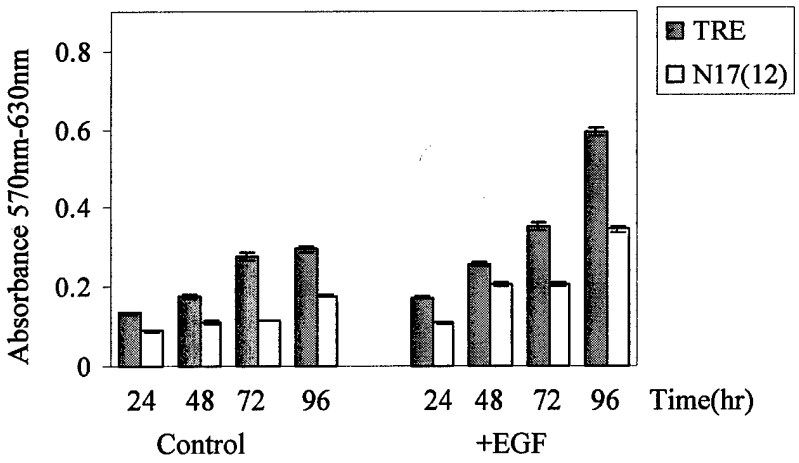
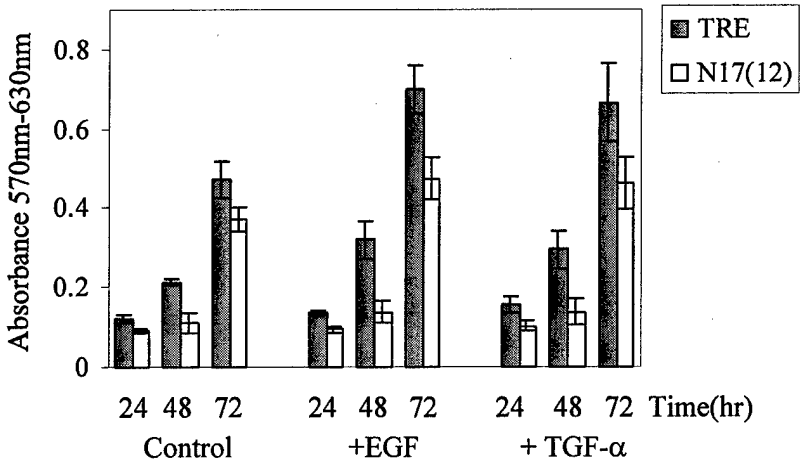

β-Casein
β-Actin
Time(hr)
0 24 48 0 24 48 0 24 48
Uninfected
Control Virus
DN-Ras Virus
β-Casein (R.U.)
0.3
0.25
0.2
0.15
0.1
0.05
0
0 24 48
Hrs DIP
Uninfected
Control Virus
DN-Ras Virus

FIG.6

A.



B.

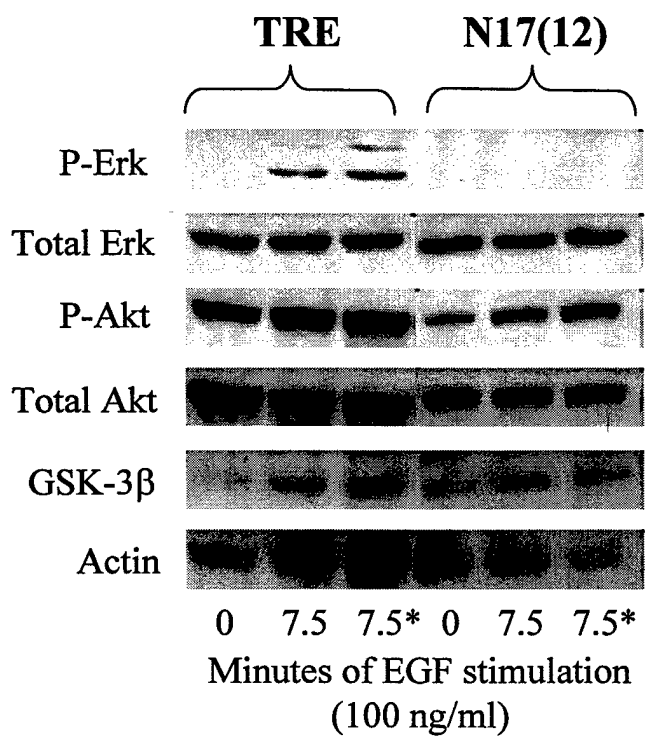


C.

Experiment #	Cell Line	% Reduction β-Casein RNA
1	TRE	99.1 %
	N17(12)	0 %
2	TRE	41.5 %
	N17(12)	0 %

FIG.7

A.


TRE
N17(12)
P-Erk
Total Erk
P-Akt
Total Akt
GSK-3β
Actin
0 7.5 7.5* 0 7.5 7.5*
Minutes of EGF stimulation
(100 ng/ml)

B.

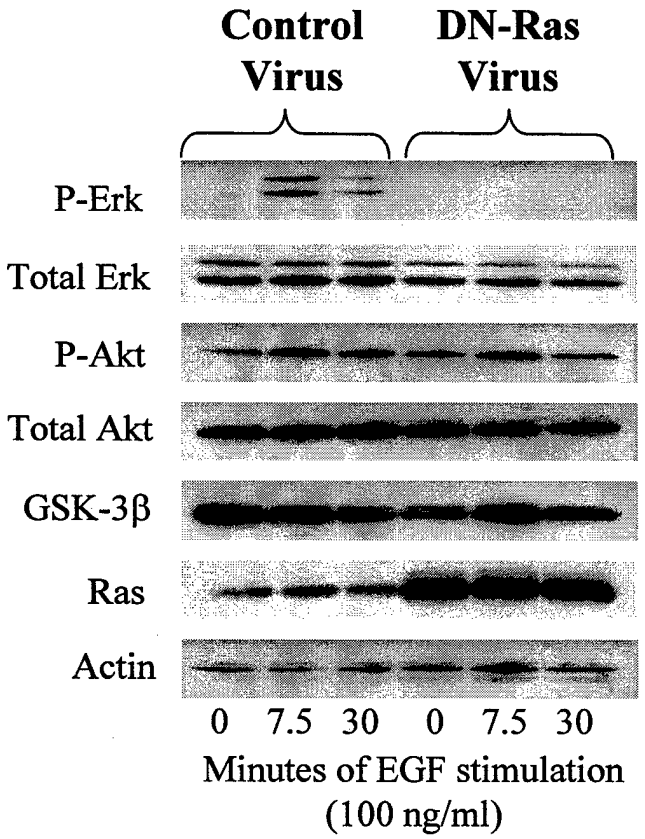

Control Virus
DN-Ras Virus
P-Erk
Total Erk
P-Akt
Total Akt
GSK-3β
Ras
Actin
0 7.5 30 0 7.5 30
Minutes of EGF stimulation
(100 ng/ml)

Figure 8. Microarray Results

1. Results of mouse 3.8K array (N=4)

accession	mean	Name
NM_010217	6.02275	connective tissue growth factor
NM_008843	4.232	prolactin induced protein
NM_013467	4.11725	alcohol dehydrogenase family 1, subfamil
NM_008489	3.9215	lipopolysaccharide binding protein
NM_008952	3.54625	peroxisomal sarcosine oxidase
NM_013515	3.34025	erythrocyte protein band 7.2
NM_009721	3.318	ATPase, Na+/K+ transporting, beta 1 poly
NM_011338	2.9665	small inducible cytokine A9
NM_012046	2.87525	sporulation protein, meiosis-specific, S
NM_011461	2.85725	Spi-C transcription factor (Spi-1/PU.1 r
NM_008065	2.6545	GA repeat binding protein, alpha
NM_007607	2.54975	carbonic anhydrase 4
NM_011361	2.399	serum/glucocorticoid regulated kinase
NM_019496	2.05575	Alport syndrome, mental retardation, mid
NM_009681	1.96225	adaptor-related protein complex AP-3, si
NM_009775	1.95425	benzodiazepine receptor, peripheral
NM_009779	1.92625	complement component 3a receptor 1
NM_013634	1.849	peroxisome proliferator activated recept
NM_013799	1.8485	arginine-tRNA-protein transferase 1
NM_011057	1.8335	platelet derived growth factor, B polype
NM_009127	1.8125	stearoyl-Coenzyme A desaturase 1
NM_007918	1.80025	eukaryotic translation initiation factor
NM_011339	1.7985	small inducible cytokine subfamily B, me
NM_010028	1.79525	DEAD (aspartate-glutamate-alanine-aspart
NM_010343	1.78875	glutathione peroxidase 5
NM_007408	1.75025	adipose differentiation related protein
NM_019946	1.74375	RIKEN cDNA 1500002K10 gene
NM_015743	1.743	nuclear receptor subfamily 4, group A, m
NM_013669	1.73625	synaptosomal-associated protein, 91 kDa
NM_009128	1.70625	stearoyl-Coenzyme A desaturase 2
NM_013698	1.6925	TXK tyrosine kinase
NM_008250	1.69075	H2.0-like homeo box gene
NM_009121	1.683	spermidine/spermine N1-acetyl transferas
NM_013602	1.675	metallothionein 1
NM_010381	1.6695	histocompatibility 2, class II antigen E
NM_011704	1.66075	vanin 1
NM_019751	1.65475	upregulated by 1,25-dihydroxyvitamin D-3
NM_010638	1.651	Kruppel-like factor 9
NM_009161	1.645	sarcoglycan, alpha (50kDa dystrophin-ass
NM_010286	1.644	glucocorticoid-induced leucine zipper
NM_007528	1.64125	BcL6-associated zinc finger protein
NM_019703	1.6345	phosphofructokinase, platelet

NM_020009	1.62975	FK506 binding protein 12-rapamycin assoc
NM_017373	1.62575	nuclear factor, interleukin 3, regulated
NM_009270	1.6235	squalene epoxidase
NM_016879	1.59425	keratin complex 2, basic, gene 18
NM_010778	1.58575	membrane cofactor protein
NM_020010	1.58375	cytochrome P450, 51
NM_019497	1.58	G protein-coupled receptor kinase 2, gro
NM_017480	1.5735	inducible T-cell co-stimulator
NM_016984	1.57275	transient receptor protein 4
NM_011997	1.567	caspase 8 associated protein 2
NM_009047	1.56675	rad and gem related GTP-binding protein
NM_007853	1.56425	degenerative spermatocyte homolog (Droso
NM_013793	1.56275	killer cell lectin-like receptor, subfam
NM_007826	1.5625	dachshund 1 (Drosophila)
NM_007752	1.55875	ceruloplasmin
NM_019699	1.55325	delta-6 fatty acid desaturase
NM_010471	1.55175	hippocalcin
NM_008467	1.5455	karyopherin (importin) alpha 4
NM_015774	1.544	ERO1-like (S. cerevisiae)
NM_011313	1.5435	calcium binding protein A6 (calcyclin)
NM_015780	1.54125	complement factor H-related protein (CFH
NM_010104	1.5375	endothelin 1
NM_009895	1.52875	cytokine inducible SH2-containing protei
NM_012032	1.5275	tumor differentially expressed 1
NM_013617	1.52675	olfactory receptor 65
NM_009312	1.5255	tachykinin 2
NM_007663	1.52475	cadherin 16
NM_011944	1.51725	mitogen activated protein kinase kinase
NM_011108	1.5155	phospholipase A2, group IIA (platelets,
NM_007996	1.51275	ferredoxin 1
NM_019814	1.51	hypoxia induced gene 1
NM_010572	1.50625	insulin receptor substrate 4
NM_010572	1.50625	insulin receptor substrate 4
NM_010362	1.50625	glutathione S-transferase like
NM_010362	1.50625	glutathione S-transferase like
NM_009529	1.50525	Xlr-related, meiosis regulated
NM_016963	1.5045	tropomodulin 3
NM_019803	1.504	ubiquitin-conjugating enzyme E2G 2
NM_013470	1.5015	annexin A3
NM_008726	1.5005	natriuretic peptide precursor type B
NM_008594	0.80525	milk fat globule-EGF factor 8 protein
NM_013870	0.8035	smoothelin
NM_011034	0.8025	peroxiredoxin 1
NM_019953	0.79725	transmembrane protein 4
NM_008619	0.79175	Moloney leukemia virus 10
NM_010368	0.79075	beta-glucuronidase structural
NM_009374	0.78	transglutaminase 3, E polypeptide

NM_007950	0.775	epiregulin
NM_011543	0.763	transcription elongation factor B (SIII)
NM_019564	0.76275	protease, serine, 11 (Igf binding)
NM_008211	0.7585	H3 histone, family 3B
NM_019774	0.7565	A kinase anchor protein 8
NM_019992	0.754	BCR downstream signaling 1
NM_016850	0.753	interferon regulatory factor 7
NM_019861	0.7505	cathepsin F
NM_008160	0.7145	glutathione peroxidase 1
NM_007729	0.7135	procollagen, type XI, alpha 1
NM_011481	0.70275	src-related kinase lacking C-terminal re
NM_011371	0.6965	sialyltransferase 7 ((alpha-N-acetylneur
NM_008410	0.69325	integral membrane protein 2 B
NM_010229	0.69175	FMS-like tyrosine kinase 3
NM_011163	0.6835	eukaryotic translation initiation factor
NM_010742	0.683	lymphocyte antigen 6 complex, locus D
NM_008210	0.662	H3 histone, family 3A
NM_008679	0.63825	nuclear receptor coactivator 3
NM_008557	0.629	FXYD domain-containing ion transport reg
NM_013473	0.60975	annexin A8
NM_013494	0.6075	carboxypeptidase E
NM_009335	0.59975	transcription factor AP-2, gamma
NM_009320	0.599	solute carrier family 6 (neurotransmitte
NM_008536	0.5975	transmembrane 4 superfamily member 1
NM_011271	0.5115	ribonuclease 1, pancreatic
NM_008764	0.41725	tumor necrosis factor receptor superfami
NM_010701	0.331	leukocyte cell derived chemotaxin 1

2. Results of NIA 15 K array (N=2)

accession	mean	name
XM_138640.1	5.96569	UNKNOWN: Similar to Mus musculus similar to CG4995 gene product [Drosophila melai
BC015260.1	5.588452	Mus musculus, FK506 binding protein 5 (51 kDa), clone MGC:18417 IMAGE:4237766, m
XM_130171.2	4.723103	Mus musculus lipocalin 2 (Lcn2), mRNA
XM_129806.2	4.382629	Mus musculus RIKEN cDNA 1700029F09 gene (1700029F09Rik), mRNA
V00711.1	4.22148	Mus musculus mitochondrial genome
NM_009160.1	4.141002	Mus musculus surfactant associated protein D (Sftpd), mRNA
	3.909285	UNKNOWN
	3.886757	UNKNOWN
NM_009647.1	3.819532	Mus musculus adenylate kinase 4 (Ak4), mRNA
BC036087.1	3.760063	Homo sapiens, similar to purine-rich element binding protein A, clone IMAGE:5284976, r
	3.478722	UNKNOWN
XM_127855.2	3.41566	Mus musculus similar to Diacylglycerol kinase, delta (Diglyceride kinase) (DGK-delta) (D.
NM_025816.1	3.381423	Mus musculus RIKEN cDNA 1200003J11 gene (1200003J11Rik), mRNA
	3.220104	UNKNOWN
	3.207818	UNKNOWN
	3.150818	UNKNOWN

L07096.1	3.145504	Mus domesticus strain MilP mitocondrion genome, complete sequence
AB042432.1	3.099236	Mus musculus domesticus mitochondrial DNA, complete genome
V00711.1	3.092504	Mus musculus mitochondrial genome
	3.09066	UNKNOWN
XM_098004.1	3.065451	UNKNOWN: Similar to Homo sapiens LOC151103 (LOC151103), mRNA
	3.034709	UNKNOWN
	3.032939	UNKNOWN
	3.012594	UNKNOWN
NM_022032.1	3.010094	Mus musculus p53 apoptosis effector related to Pmp22 (Perp-pending), mRNA
	2.973903	UNKNOWN
NM_008489.1	2.968955	Mus musculus lipopolysaccharide binding protein (Lbp), mRNA
	2.95416	UNKNOWN
V00711.1	2.951182	Mus musculus mitochondrial genome
	2.886258	UNKNOWN
	2.872857	UNKNOWN
BC011070.1	2.804611	UNKNOWN: Similar to Mus musculus, clone MGC:19437 IMAGE:3497896, mRNA, comp
	2.802611	UNKNOWN
NM_010816.1	2.765922	UNKNOWN: Similar to Mus musculus microrchidia (Morc), mRNA
	2.757215	UNKNOWN
XM_126337.1	2.74768	Mus musculus similar to KIAA0774 protein (2210021E03Rik), mRNA
XM_129146.1	2.72966	Mus musculus RIKEN cDNA 2410004D18 gene (2410004D18Rik), mRNA
	2.702827	UNKNOWN
S79304.1	2.669833	UNKNOWN: Similar to Rattus sp. cytochrome oxidase subunit I mRNA, partial cds; and t
NM_133753.1	2.65729	Mus musculus RIKEN cDNA 1300002F13 gene (1300002F13Rik), mRNA
	2.620403	UNKNOWN
NM_008110.1	2.612164	Mus musculus growth differentiation factor 9 (Gdf9), mRNA
XM_131651.1	2.603165	Mus musculus RIKEN cDNA 1500001L03 gene (1500001L03Rik), mRNA
	2.559403	UNKNOWN
XM_129146.1	2.548471	Mus musculus RIKEN cDNA 2410004D18 gene (2410004D18Rik), mRNA
AC108508.2	2.530429	Genomic sequence for Mus musculus, clone RP23-437O14, complete sequence
BC012020.1	2.495217	Mus musculus, Similar to cytochrome c oxidase III, mitochondrial, clone IMAGE:4500967
	2.486124	UNKNOWN
NM_027464.1	2.484398	Mus musculus RIKEN cDNA 5730469M10 gene (5730469M10Rik), mRNA
XM_125538.2	2.44506	Mus musculus RIKEN cDNA 1110002G11 gene (1110002G11Rik), mRNA
NM_033075.1	2.432925	Mus musculus G7e protein (G7e-pending), mRNA
	2.427377	UNKNOWN
	2.42539	UNKNOWN
NM_007691.1	2.412595	Mus musculus checkpoint kinase 1 homolog (S. pombe) (Chek1), mRNA
NM_133737.1	2.408001	Mus musculus RIKEN cDNA 1700003F10 gene (1700003F10Rik), mRNA
NM_145632.1	2.384494	Mus musculus similar to PROBABLE DNA-DIRECTED RNA POLYMERASES I, II, AND
	2.377329	UNKNOWN
AB042432.1	2.369087	UNKNOWN: Similar to Mus musculus domesticus mitochondrial DNA, complete genome
AL117595.1	2.365093	UNKNOWN: Similar to Homo sapiens mRNA; cDNA DKFZp564C2063 (from clone DKFZ
AB042523.1	2.359226	UNKNOWN: Similar to Mus musculus mitochondrial DNA, complete genome, strain:SAM
XM_153383.1	2.33976	UNKNOWN: Similar to Mus musculus LOC217571 (LOC217571), mRNA
NM_019714.1	2.338437	Mus musculus B lymphocyte gene 1 (Bce1-pending), mRNA
	2.330001	UNKNOWN
XM_181325.1	2.328126	Mus musculus RIKEN cDNA 2310063P06 gene (2310063P06Rik), mRNA

AB042432.1	2.327995	Mus musculus domesticus mitochondrial DNA, complete genome
BC029250.1	2.324261	Mus musculus, clone IMAGE:4225337, mRNA
AF214115.1	2.314126	UNKNOWN: Similar to Peromyscus maniculatus H19 mRNA, complete cds
XM_138680.1	2.309929	UNKNOWN: Similar to Mus musculus similar to Zinc finger protein 208 (LOC212569), mF
XM_178097.1	2.306275	UNKNOWN: Similar to Mus musculus similar to zinc finger protein 97 [Mus musculus] (L
	2.300626	UNKNOWN
	2.300514	UNKNOWN
BC005741.1	2.299709	UNKNOWN: Similar to Mus musculus, clone MGC:11932 IMAGE:3599820, mRNA, comp
AB042432.1	2.290126	Mus musculus domesticus mitochondrial DNA, complete genome
AF378830.1	2.280251	Mus musculus cytochrome c oxidase subunit II (Cox2) mRNA, complete cds; mitochondı
NM_133689.1	2.269086	Mus musculus RIKEN cDNA 4930579J09 gene (4930579J09Rik), mRNA
NM_010937.1	2.263036	Mus musculus neuroblastoma ras oncogene (Nras), mRNA
NM_011340.1	2.258001	Mus musculus serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigı
NM_019946.1	2.255483	Mus musculus microsomal glutathione S-transferase 1 (Mgst1), mRNA
Y17323.1	2.251001	UNKNOWN: Similar to Rattus norvegicus CDK109 mRNA
X70496.1	2.242086	UNKNOWN: Similar to R. norvegicus mRNA for Mss4 protein
	2.231822	UNKNOWN
	2.229957	UNKNOWN
BC031203.1	2.229787	Mus musculus, clone IMAGE:5066398, mRNA
NM_010638.1	2.226703	UNKNOWN: Similar to Mus musculus Kruppel-like factor 9 (Klf9), mRNA
	2.222709	UNKNOWN
XM_129811.1	2.222592	Mus musculus RIKEN cDNA 2310061I09 gene (2310061I09Rik), mRNA
NM_009681.1	2.21833	Mus musculus adaptor-related protein complex AP-3, sigma 1 subunit (Ap3s1), mRNA
XM_150228.2	2.208729	UNKNOWN: Similar to Mus musculus LOC235584 (LOC235584), mRNA
	2.20785	UNKNOWN
BC006888.1	2.198633	UNKNOWN: Similar to Mus musculus, clone MGC:6272 IMAGE:2647757, mRNA, compl
	2.198065	UNKNOWN
BC012020.1	2.190789	Mus musculus, Similar to cytochrome c oxidase III, mitochondrial, clone IMAGE:4500967
	2.184586	UNKNOWN
XM_134035.1	2.176983	Mus musculus similar to hypothetical protein FLJ11305 [Homo sapiens] (LOC234069), r
NM_146163.1	2.175514	Mus musculus hypothetical protein MGC11654 (MGC11654), mRNA
	2.175471	UNKNOWN
	2.170787	UNKNOWN
XM_123496.1	2.166452	Mus musculus RIKEN cDNA 1810030F05 gene (1810030F05Rik), mRNA
BC012020.1	2.15968	Mus musculus, Similar to cytochrome c oxidase III, mitochondrial, clone IMAGE:4500967
	2.158898	UNKNOWN
X70496.1	2.155551	UNKNOWN: Similar to R. norvegicus mRNA for Mss4 protein
X17502.1	2.155318	Mouse mRNA overexpressed and amplified in teratocarcinoma cell line ECA39
	2.151456	UNKNOWN
	2.147605	UNKNOWN
	2.14696	UNKNOWN
BC032201.1	2.143287	UNKNOWN: Similar to Mus musculus, clone MGC:35617 IMAGE:5342197, mRNA, comp
	2.129659	UNKNOWN
XM_150141.2	2.119454	Mus musculus LOC234574 (LOC234574), mRNA
NM_021552.1	2.114036	Mus musculus RIKEN cDNA 5730427N09 gene (5730427N09Rik), mRNA
XM_145612.1	2.108571	Mus musculus similar to MrgB2 G protein-coupled receptor [Mus musculus] (LOC24397
	2.107172	UNKNOWN
XM_112239.1	2.097092	UNKNOWN: Similar to Mus musculus similar to zinc finger protein 40 (LOC195533), mRI

	2.096235	UNKNOWN
NM_009266.1	2.095076	Mus musculus selenophosphate synthetase 2 (Sps2), mRNA
NM_031386.1	2.091199	Mus musculus testis expressed gene 14 (Tex14), mRNA
XM_158024.1	2.084281	UNKNOWN: Similar to Mus musculus LOC214101 (LOC214101), mRNA
XM_144763.1	2.08246	UNKNOWN: Similar to Mus musculus similar to anti-poly(dC) monoclonal antibody kappa
NM_009263.1	2.080536	Mus musculus secreted phosphoprotein 1 (Spp1), mRNA
	2.079527	UNKNOWN
NM_011258.1	2.067001	Mus musculus replication factor C, 140 kDa (Recc1), mRNA
XM_129401.2	2.066203	Mus musculus RIKEN cDNA 4933411J24 gene (4933411J24Rik), mRNA
XM_138798.1	2.056712	UNKNOWN: Similar to Mus musculus similar to pol protein [Sus scrofa] (LOC238787), m
NM_007621.1	2.049525	Mus musculus carbonyl reductase 2 (Cbr2), mRNA
	2.041495	UNKNOWN
NM_024185.2	2.039376	Mus musculus RIKEN cDNA 2310047O13 gene (2310047O13Rik), mRNA
NM_013449.1	2.037027	UNKNOWN: Similar to Homo sapiens bromodomain adjacent to zinc finger domain, 2A (I
	2.032549	UNKNOWN
NM_015263.1	2.031245	Homo sapiens rabconnectin-3 (RC3), mRNA
NM_007621.1	2.020431	Mus musculus carbonyl reductase 2 (Cbr2), mRNA
NM_007570.1	2.018471	Mus musculus B-cell translocation gene 2, anti-proliferative (Btg2), mRNA
XM_128441.2	2.007491	Mus musculus similar to Zinc finger protein 118 [Mus musculus] (LOC224591), mRNA
	2.005111	UNKNOWN
	2.001626	UNKNOWN
NM_033584.1	0.500316	Mus musculus protocadherin gamma subfamily A, 1 (Pcdhga1), mRNA
XM_134192.1	0.496781	UNKNOWN: Similar to Mus musculus RIKEN cDNA 2410003B16 gene (2410003B16Rik)
NM_080467.1	0.49677	Mus musculus ATPase, H+ transporting, lysosomal V0 subunit A isoform 4 (Atp6v0a4), r
XM_132528.1	0.496626	Mus musculus similar to Activator 1 38 kDa subunit (Replication factor C 38 kDa subunit
NM_011595.1	0.494771	Mus musculus tissue inhibitor of metalloproteinase 3 (Timp3), mRNA
NM_008894.1	0.493414	Mus musculus polymerase (DNA directed), delta 2, regulatory subunit (50 kDa) (Pold2), r
NM_011638.1	0.493063	Mus musculus transferrin receptor (Trfr), mRNA
	0.492591	UNKNOWN
XM_134396.1	0.492277	Mus musculus G protein-coupled receptor 56 (Gpr56), mRNA
NM_013870.1	0.492239	Mus musculus smoothelin (Smtn), mRNA
NM_134189.1	0.491	Mus musculus UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminy
NM_019813.1	0.490641	Mus musculus drebrin 1 (Dbn1), mRNA
XM_128833.1	0.490383	Mus musculus RIKEN cDNA 0610011L04 gene (0610011L04Rik), mRNA
	0.48763	UNKNOWN
	0.487417	UNKNOWN
XM_129697.2	0.486006	Mus musculus RIKEN cDNA 3110069K09 gene (3110069K09Rik), mRNA
NM_008496.1	0.484992	Mus musculus lectin, galactose binding, soluble 7 (Lgals7), mRNA
NM_009828.1	0.484646	Mus musculus cyclin A2 (Ccna2), mRNA
XM_126327.1	0.483836	Mus musculus RIKEN cDNA 1500005K14 gene (1500005K14Rik), mRNA
NM_009255.1	0.483379	Mus musculus serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen acti
NM_019521.1	0.483139	Mus musculus growth arrest specific 6 (Gas6), mRNA
NM_146098.1	0.482955	Mus musculus hypothetical protein MGC28180 (MGC28180), mRNA
	0.481381	UNKNOWN
XM_137043.1	0.480612	Mus musculus similar to squamous cell carcinoma antigen recognized by T cells 2 [Hom
NM_013737.1	0.478506	Mus musculus phospholipase A2 group VII (platelet-activating factor acetylhydrolase, pla
BC023928.1	0.473398	Mus musculus, clone IMAGE:5324278, mRNA
NM_023526.1	0.4712	Mus musculus RIKEN cDNA 2400004O09 gene (2400004O09Rik), mRNA

	0.470124	UNKNOWN
BC030860.1	0.468683	Mus musculus, Similar to AT2 receptor-interacting protein 1, clone IMAGE:4206405, mR
	0.468363	UNKNOWN
NM_009416.2	0.466203	Mus musculus tropomyosin 2, beta (Tpm2), mRNA
XM_126935.1	0.458185	Mus musculus RIKEN cDNA 2310009M18 gene (2310009M18Rik), mRNA
NM_008512.1	0.455506	Mus musculus low density lipoprotein receptor-related protein 1 (Lrp1), mRNA
NM_024427.1	0.455214	Mus musculus tropomyosin 1, alpha (Tpm1), mRNA
NM_007564.1	0.455199	Mus musculus zinc finger protein 36, C3H type-like 1 (Zfp36l1), mRNA
NM_013541.1	0.454435	Mus musculus glutathione S-transferase, pi 2 (Gstp2), mRNA
NM_133185.1	0.453914	Mus musculus RIKEN cDNA 0610011C19 gene (0610011C19Rik), mRNA
XM_132426.1	0.450573	Mus musculus RIKEN cDNA 2700063G02 gene (2700063G02Rik), mRNA
NM_016957.1	0.449876	Mus musculus high mobility group nucleosomal binding domain 2 (Hmgn2), mRNA
XM_128606.2	0.449185	Mus musculus similar to hypothetical protein DKFZp761D0211 [Homo sapiens] (LOC215
NM_007680.1	0.448885	Mus musculus Eph receptor B6 (Ephb6), mRNA
	0.447048	UNKNOWN
	0.445702	UNKNOWN
NM_007899.1	0.444352	UNKNOWN: Similar to Mus musculus extracellular matrix protein 1 (Ecm1), mRNA
XM_129745.2	0.443464	Mus musculus procollagen, type III, alpha 1 (Col3a1), mRNA
XM_181394.1	0.443004	Mus musculus procollagen, type IX, alpha 3 (Col9a3), mRNA
NM_009929.1	0.442813	Mus musculus procollagen, type XVIII, alpha 1 (Col18a1), mRNA
BC006061.1	0.442602	Mus musculus, clone IMAGE:3591705, mRNA
NM_023118.1	0.441519	Mus musculus disabled homolog 2 (Drosophila) (Dab2), mRNA
NM_007833.1	0.440749	UNKNOWN: Similar to Mus musculus decorin (Dcn), mRNA
NM_025378.1	0.44	Mus musculus RIKEN cDNA 1110004C05 gene (1110004C05Rik), mRNA
XM_128198.1	0.43996	Mus musculus expressed sequence AI316867 (AI316867), mRNA
XM_126577.2	0.439568	Mus musculus mitogen activated protein kinase kinase 6 (Map2k6), mRNA
NM_013467.1	0.439396	Mus musculus aldehyde dehydrogenase family 1, subfamily A1 (Aldh1a1), mRNA
	0.438683	UNKNOWN
	0.437093	UNKNOWN
XM_132709.1	0.436531	Mus musculus RIKEN cDNA 2310008J16 gene (2310008J16Rik), mRNA
AL161729.27	0.435648	UNKNOWN: Similar to Human DNA sequence from clone RP11-435O5 on chromosome
XM_132915.2	0.430084	Mus musculus similar to retinoic acid inducible protein 3 [Mus musculus] (LOC232431), r
	0.428727	UNKNOWN
NM_146098.1	0.426666	Mus musculus hypothetical protein MGC28180 (MGC28180), mRNA
NM_146102.1	0.414826	Mus musculus hypothetical protein MGC28084 (MGC28084), mRNA
NM_008160.1	0.41452	Mus musculus glutathione peroxidase 1 (Gpx1), mRNA
	0.407415	UNKNOWN
NM_019791.1	0.406249	Mus musculus melanoma antigen, family D, 1 (Maged1), mRNA
XM_134149.2	0.406008	Mus musculus similar to FAT tumor suppressor (Drosophila) homolog [Rattus norvegicus
	0.402814	UNKNOWN
XM_137731.2	0.397649	Mus musculus similar to Component of gems 4 (Gemin4) (p97) (LOC237853), mRNA
NM_010500.1	0.396203	Mus musculus immediate early response 5 (Ier5), mRNA
M95495.1	0.394583	UNKNOWN: Similar to Dog Na/Cl-dependent taurine transporter mRNA, complete cds
NM_144865.1	0.389533	Mus musculus hypothetical protein MGC28827 (MGC28827), mRNA
BC034188.1	0.386053	Mus musculus, clone IMAGE:4976037, mRNA
NM_138685.1	0.37926	Mus musculus elafin-like protein I (SWAM1), mRNA
NM_053178.1	0.377991	Mus musculus lipidosis-related protein lipidosin (lpd), mRNA
AB085694.1	0.367999	Mus musculus bpag1-e mRNA for bullous pemphigoid antigen 1-e, partial cds

NM_022880.1	0.359247	Mus musculus solute carrier family 29 (nucleoside transporters), member 1 (Slc29a1), m
NM_011666.1	0.359071	Mus musculus ubiquitin-activating enzyme E1C (Ube1c), mRNA
NM_016917.1	0.352644	Mus musculus solute carrier family 39 (iron-regulated transporter), member 1 (Slc39a1),
XM_146260.2	0.348582	UNKNOWN: Similar to Mus musculus similar to hypothetical protein FLJ10408 [Homo sa
NM_018778.1	0.335626	Mus musculus claudin 8 (Cldn8), mRNA
NM_007833.1	0.332508	Mus musculus decorin (Dcn), mRNA
	0.331149	UNKNOWN
NM_008557.1	0.315307	Mus musculus FXYD domain-containing ion transport regulator 3 (Fxyd3), mRNA
D45203.1	0.287273	Mouse pentylenetetrazol-related mRNA PTZ-17 (3'UTR of E3.1)
	0.271005	UNKNOWN
NM_007631.1	0.254491	Mus musculus cyclin D1 (Ccnd1), mRNA
NM_014840.1	0.238303	UNKNOWN: Similar to Homo sapiens KIAA0537 gene product (KIAA0537), mRNA
NM_008086.1	0.207099	Mus musculus growth arrest specific 1 (Gas1), mRNA
BC031421.1	0.120346	Mus musculus, clone IMAGE:3983988, mRNA

3. Comparison of common genes from two arrays for reproducibility. Results from two arrays were compared for reproducibility, data showed that the regulation of genes have the same direction (up- or down-regulation), although the mean numbers were different.

3.8 K mean	accession number	NIA mean	Name
3.9215	NM_008489	2.968955	lipopolysaccharide binding protein
2.399	NM_011361	1.906995	serum/glucocorticoid regulated kinase
1.96225	NM_009681	2.21833	adaptor-related protein complex AP-3, si
1.75025	NM_007408	1.27183	adipose differentiation related protein
1.74375	NM_019946	2.255483	RIKEN cDNA 1500002K10 gene
1.70625	NM_009128	1.425331	stearoyl-Coenzyme A desaturase 2
1.683	NM_009121	1.646836	spermidine/spermine N1-acetyl transferas
1.651	NM_010638	2.226703	Kruppel-like factor 9
1.6235	NM_009270	1.325294	squalene epoxidase
1.567	NM_011997	1.842824	caspase 8 associated protein 2
1.56425	NM_007853	1.735051	degenerative spermatocyte homolog (Droso
1.5435	NM_011313	1.32803	calcium binding protein A6 (calcyclin)
1.5045	NM_016963	1.377895	tropomodulin 3
1.504	NM_019803	1.256886	ubiquitin-conjugating enzyme E2G 2
1.5005	NM_008726	1.299737	natriuretic peptide precursor type B
0.79725	NM_019953	0.570623	transmembrane protein 4
0.79725	NM_019953	0.56644	transmembrane protein 4
0.79175	NM_008619	0.864599	Moloney leukemia virus 10
0.79175	NM_008619	0.555623	Moloney leukemia virus 10
0.7585	NM_008211	0.730108	H3 histone, family 3B
0.7585	NM_008211	0.68806	H3 histone, family 3B
0.7565	NM_019774	0.984505	A kinase anchor protein 8

0.7145	NM_008160	0.41452	glutathione peroxidase 1
0.69325	NM_008410	0.620398	integral membrane protein 2 B
0.69325	NM_008410	0.570781	integral membrane protein 2 B
0.629	NM_008557	0.315307	FXYD domain-containing ion transport reg
0.59975	NM_009335	0.609136	transcription factor AP-2, gamma

Fig.9 Up-regulated genes

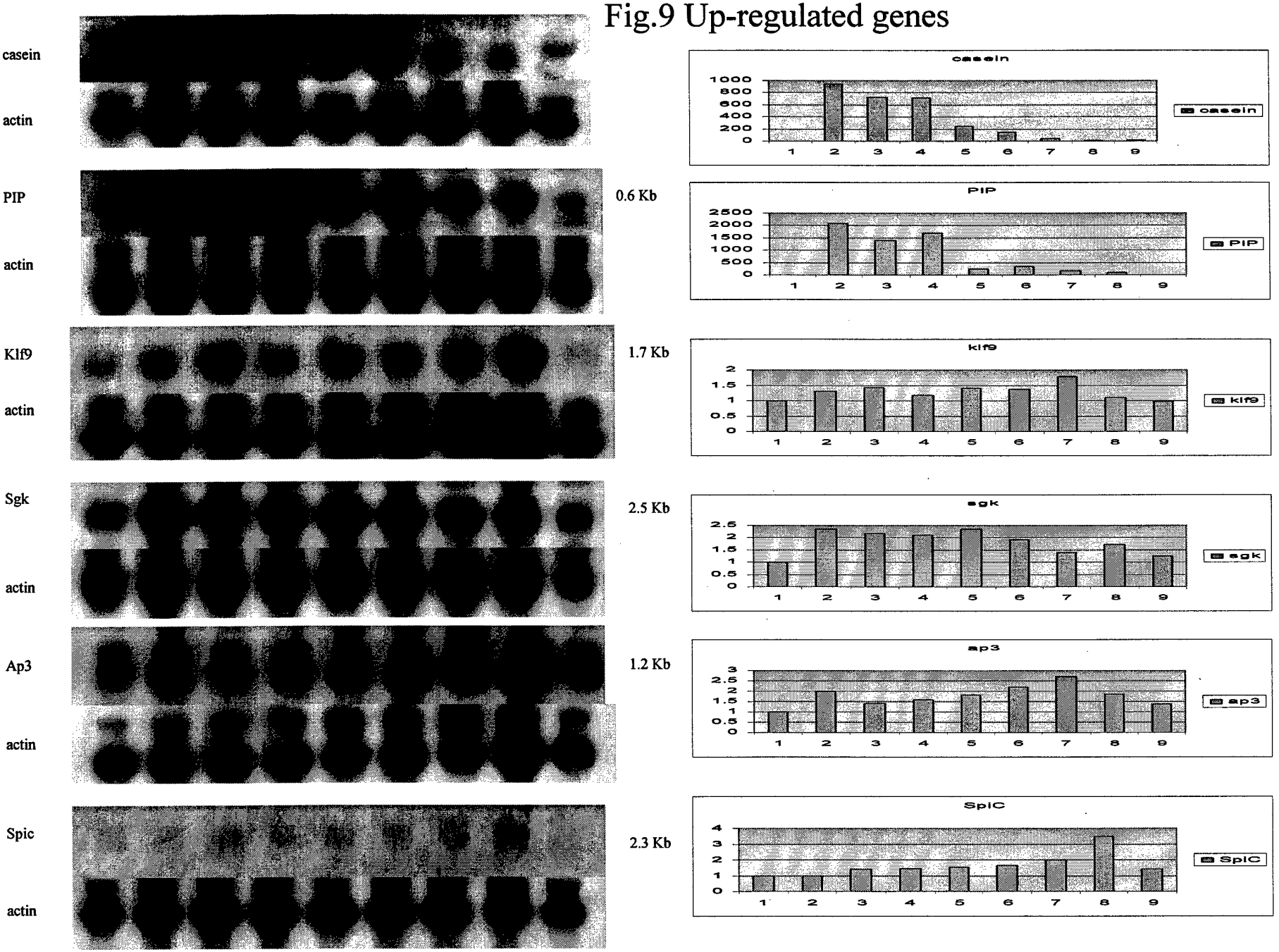
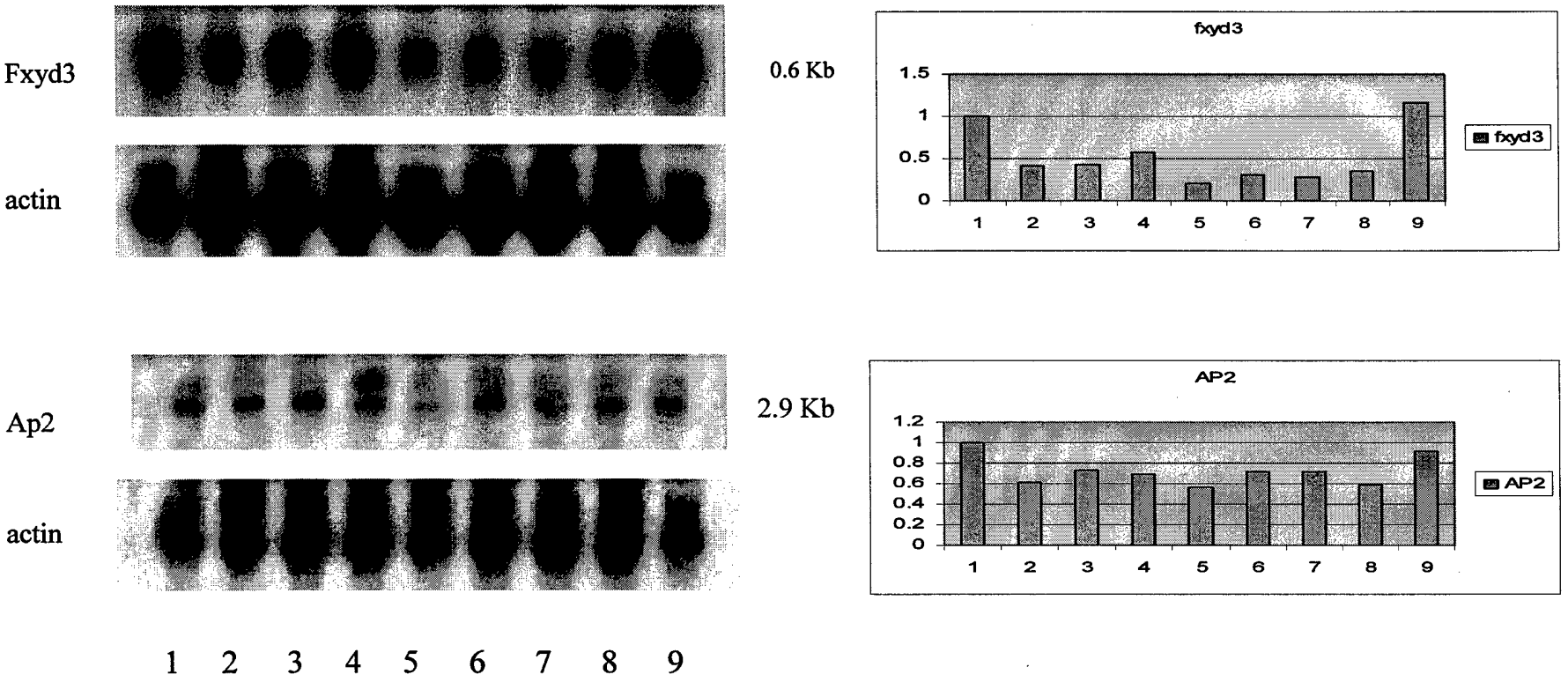


Fig. 10 Down-regulated genes



Lane 1 to 9: control, 12 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, 144 h control; Left: Northern blot of different genes and their correspondent beta-actin expression, right: fold changes of gene expression normalized by beta-actin. Abbreviation Klf9 is changed to Bteb1 in the new version of NCBI web.